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In vitro cell specific effects of rooibos (*Aspalathus linearis*) and the active compound aspalathin on malignant and benign prostate cell lines

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Abstract Rooibos (*A. linearis*) may promote oxidative stress in malignant prostate cells through increased reactive oxygen species (ROS) production but may protect normal prostate cells from ROS induced stress. To test this hypothesis, malignant LNCaP and benign RWPE-1 prostate cells were exposed to aqueous solutions of 125 - 50 000 µg/mL fermented rooibos, unfermented rooibos or aspalathin for 72-hours. ROS quantification using CM-H_2DCFDA fluorophore produced significantly ($P < 0.05$) increased ROS levels in malignant (LNCaP) prostate cells but not in benign (RWPE-1) prostate cells. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay using mitochondrial dehydrogenase activity as a function of cell viability showed a concentration-dependent, significant ($P < 0.05$) decrease in viability of LNCaP cells following treatment with 5 000 - 50 000 µg/mL unfermented rooibos whilst in RWPE-1, viability was maintained at all concentrations used. The apoptotic assay using annexin V showed significantly ($P < 0.05$) increased percentage of apoptotic and necrotic LNCaP cells, respectively compared with their respective negative controls. In contrast, benign RWPE-1 cells produced a significant ($P < 0.05$) drop in the percentage of apoptotic and necrotic cells, respectively compared to their specific negative controls. Fermented rooibos, unfermented rooibos, and aspalathin respectively, significantly ($P < 0.05$) reduced the total serum prostate specific antigen (PSA) in malignant LNCaP prostate cells whereas in benign RWPE-1 prostate cells, the total serum PSA level was comparable to that of its specific negative control. This study suggests cytotoxic effects of unfermented rooibos on malignant LNCaP cells and protective effects on benign RWPE-1 cells against ROS induced stress. The decrease in total serum PSA seems to demonstrate possible therapeutic effects of *A. linearis* on malignant LNCaP prostate cells and possible maximum health benefits from total polyphenol content.

Keywords: *Apoptosis, necrosis cytotoxicity, rooibos, prostate specific antigen (PSA), reactive oxygen species (ROS).*

Introduction

Prostate cancer is one of the major health challenges men are facing. Presently, it is the second most leading cause of death in men amongst all cancers worldwide (Bray et al., 2018). Testosterone or dihydrotestosterone often stimulates prostate cancer growth which is why cancer management involves use of pharmaceuticals that promote androgen withdrawal or that inhibit androgen receptors (Perlmutter and Lepor, 2007; Spratt et al., 2021). Androgen deprivation therapy as a cancer management approach is aimed at impairing androgen production and castrating circulating levels of testosterone through reduction of AR ligand availability and subsequent AR-mediated increase in prostate cell production. This can involve orchiectomy, chronic administration of gonadotropin-releasing hormone (GnRH) agonists or estrogen therapy (Harris et al., 2009). Radical prostatectomy and radiotherapy are also used. Unfortunately, some patients may experience complications that may include erectile dysfunction, urinary incontinence, rectal bleeding and lymphedema among others (Smith-Palmer, Takizawa and Valentine, 2019). Most prostate cancers develop very slowly, which allows for alternative treatment options such as traditional herbs. Herbal medicines possess active compounds with anti-oxidative and superoxide scavenging properties which can inhibit lipid peroxidation and cancer development (Ho, Leung and Chan, 2002). The long period involved with the development of cancer provides an opportunity for herbal medicines to work as cancer blocking (Russo et al., 2017) and suppressing agents (Surh, 2003) thereby preventing its initiation, promotion and progression. Cancer blocking agents prevent carcinogens from initiating cancer through enhancement of carcinogen detoxification, modification of carcinogen uptake and metabolism, scavenging of reactive oxygen species (ROS) and enhancement of DNA repair. Cancer-suppressing agents inhibit cancer promotion and progression after the formation of pre-neoplastic cells by interfering with cell cycle regulation, signal transduction, transcriptional regulation, and apoptosis (Msiska, 2015). In line with this, a number of health benefits from antioxidant effects of flavonoids and phenolic acids present in different herbal teas have been reported, as demonstrated by their apoptosis-inducing effects in cancer cell lines (Tungmunnithum et al., 2018). An herbal product, PC-SPES, has been reported to prevent proliferation of prostate cancer cell lines both in vitro and in vivo and has the potential to protect cells from gene mutation and differentiation. The herbal formulation may also down-regulate the androgen receptor, induce apoptosis via inhibition of the bcl-2 gene, and up-regulate the expression of p53 (Marks et al., 2002). Similarly, lycopene, an active compound found in tomato, modulate cancer metabolism, hormonal balance, and transcriptional activity, regulate the cell-cycle, initiate apoptosis, and prevent inflammation, angiogenesis and metastasis (Trejo-Solís et al., 2013).

To this effect, rooibos, *Aspalathus linearis,* a caffeine-free tisane with dietary antioxidants such as flavonoids, dihydrochalcone glucoside, and aspalathin (Hong, 2014) has been reported to prevent chemically induced liver damage, inflammation, lipid oxidation, hyperglycemia and oxidative stress (Hong, Lee and Kim, 2014). It has also been reported to prevent gene mutations, modulate cancer and regenerate coenzyme Q10 (Kucharska et al., 2004). Some of these effects have been attributed to its antioxidant activity that enables it to scavenge free radicals and reduce reactive oxygen species (Ajuwon, Marnewick and Davids, 2015). High levels of intracellular reactive oxygen species and a compromised antioxidant defense system are generally associated with all cancers (Liou and Storz, 2010; Waisundara, Hoon and Hoon, 2015).

Prostate-specific antigen (PSA), on the other hand, is a serine protease that is secreted exclusively by prostatic epithelial cells, and its serum concentration is elevated in men with benign prostatic hyperplasia and prostate cancer. Serum PSA measurement is one of the markers for monitoring the progression of prostate cancer and its response to therapy (Hayes and Barry, 2014). Some researchers reported a significant decrease in PSA in men with prostate cancer following treatment with (-) epigallocatechin-3-gallate (EGCG), an active compound in green tea (McLarty et al., 2009). Generally, a reduction in PSA levels may be a good pointer for apoptotic death of cancerous cells.

In vitro studies involving EGCG (Polyphenol E) on prostate cell lines have been implicated in the induction of apoptosis both in androgen-sensitive

and insensitive prostate cancer cells (Miyata et al., 2019) besides inhibiting cancer cell proliferation (Chuu et al., 2009). For this study, lymph node carcinoma of the prostate (LNCaP) and RWPE-1 cell lines were used. LNCaP is a human prostate cancer cell line similar in characteristics to those found in the stratified epithelium of the prostate gland, which expresses androgen receptors (AR) and prostate specific antigen (Heer, 2011; Namekawa et al., 2019).

The benign RWPE-1 prostate cells are similar to the normal prostatic epithelial cells in their response to growth factors, expression of PSA and androgen receptor in response to androgen exposure (Cunningham and You, 2015; Cheung et al., 2005).

However, there is no information on the effects of fermented, unfermented rooibos or aspalathin on prostate cells. Therefore, this study endeavored to investigate the effects of *A. linearis* and its active chemical compound, aspalathin, on malignant LNCaP and benign RWPE-1 prostate cells.

Materials and methods

Research study design

The research study design was analytical in nature and it involved wet laboratory experiments. Both normal and malignant prostate cancer cell lines were exposed to fermented, unfermented rooibos herbal tea extracts and their standard, aspalathin.

Ethical Clearance

This study was done in line with the principles of laboratory care developed by the Ethics Committee of University of the Western Cape, South Africa with the Ethics approval no: 13/2/37.

Plant extracts

Unfermented and fermented rooibos *(A. linearis*) was a donation from Rooibos Ltd (Clanwilliam, South Africa). Aspalathin was obtained from HWI Analytik GMBH (Rueizheim, Germany). Tisane was prepared by adding 5g of rooibos to 100 mL of freshly boiled tap water for 5 min (Opuwari, 2015). A filtrate was obtained using cheese cloth, Whatman's filter paper numbers 4 and 1, respectively and a vacuum system. Thereafter, the aqueous extracts were freeze dried and reconstituted in RPMI 1640 medium (Biochrom, Berlin, Germany) and keratinocyte serum free medium (Gibco, Germany), respectively.

Cell culture

A benign human prostate epithelial cell line (RWPE-1 CRL 11609) and malignant prostate cell line (LNCaP CRL 1740) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). RWPE-1 cells were maintained in keratinocyte serum free medium supplemented with 10 % bovine serum and epidermal growth factor (Gibco, Germany) whereas the LNCaP cells were maintained in RPMI 1640 medium (Gibco, Germany) supplemented with 10 % foetal bovine serum (FBS) (Biochrom, Berlin, Germany). To prevent contaminants growth, both cell lines were treated with penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37 °C, in humidified atmospheric air supplemented with 5 % CO_2. Following which, both cell lines were incubated in varying concentrations of fermented rooibos, unfermented rooibos or aspalathin (125, 250, 500, 1 000, 5 000, 10 000 or 50 000 µg /mL medium, respectively).

Cell viability

To assess the effects of *A. linearis* on RWPE-1 and malignant LNCaP cell viability, the MTT assay, which reflects the activity of mitochondrial dehydrogenases, was used (Monsees et al., 2000). In this regard, log-phase prostate cells were seeded into 96-well plates at a concentration of 3 000 (RWPE-1) and 2 000 (LNCaP) cells per 200 µL culture medium per well for 48 hours. Thereafter, the medium was removed, washed in 1 mL PBS, before exposing the adherent cells to seven different concentrations (125 - 50,000 µg/mL) of the extracts, aspalathin, or 6 % DMSO (positive control) in complete culture medium for 72-hours. Cell viability was quantified by removing the supernatants from all the wells. Following which, 20 µL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, Steinheim, Germany), 1 mg/mL in PBS (Oxoid, Hampshire, England) was added to each well and incubated for 4 hours at 37 °C. After removing the supernatants 100 µL of DMSO was then added to each well to dissolve the precipitated dye. After gentle shaking for 10 minutes, absorbance of the dye was measured at a wavelength of 560 nm with a background subtraction of 750 nm using an ELISA reader (Thermo electron corporation, South Africa).

Determination of ROS levels.

The protocol used in this ROS assay involved dissolving 50 µg CM-H_2DCFDA in 150 µL DMSO. This stock solution was used to prepare 12.36 mL of 7 µM CM-H_2DCFDA. RWPE-1 positive control cells were treated with 100 µM

H_2O_2 for 10 hours. LNCaP positive control cells were treated with 50 µM hydrogen peroxide (H_2O_2) for 30 minutes. Negative control RWPE-1 and LNCaP cells were incubated in keratinocyte serum free medium and RPMI medium only, respectively. LNCaP cells incubated with rooibos for 72-hour (3 000 cells per mL) were harvested using 0.25 % trypsin into 15 mL centrifuge tubes, spun down for 6 minutes at 1500 rpm. The pellet formed was then re-suspended in 400 µL PBS containing 7µM CM-H_2DCFDA and incubated at 37°C in the dark for 30 minutes to load the dye. This was followed by the addition of 4 mL PBS to the tube in order to remove excess dye. The tube was then centrifuged at 1500 rpm for 6 minutes. The supernatant was discarded and the pellet re-suspended in 400 µL PBS before transferring to 2.0 mL eppendorf tubes for analysis by flow cytometry. The fluorescence was monitored using the FL 1 channel of the BD accuri 6 Flow cytometer (Ann Arbor, MI, USA). The excitation frequency was between 492 and 495nm whereas the emission frequency was between 517 and 527nm. Untreated cells not loaded with dye were used as a negative control.

Number of apoptotic and necrotic cells

Malignant LNCaP and benign RWPE-1 cells were seeded at a density of 3 000 cells per mL of complete RPMI 1640 and keratinocyte serum free medium, respectively, for 48-hours in a 24-well plate. Cells were then exposed to 1 000 µg/mL of fermented, unfermented rooibos or aspalathin. After discarding the culture medium, cells were washed with 1 mL PBS and trypsinated with 250 µL of 0.25% Trypsin/EDTA. Thereafter, cell pellet was formed and subsequently re-suspended in 200 mL of binding buffer. Annexin V staining was accomplished following the product instruction (Clontech, Palo Alto, CA). In brief, 5 µL Alexa Fluor® 488 annexin V (Component A) and 1 µL 100 µg/mL Propidium Iodide (PI) working solution were added to each 100 µL of cell suspension; cells were incubated at room temperature for 15 minutes. After the incubation period, 400 µL 1x annexin-binding buffer was added and mixed gently. All the samples were kept on ice and then immediately taken for flow cytometry analysis using a fluorescence emission at 530 nm (green) for the FL1 channel and 585 nm (red) for the FL3 channel. The population of cells was separated into three groups: live cells with low levels of fluorescence, apoptotic cells with green fluorescence, and dead cells with both red and green fluorescence.

Determination of prostate specific antigen (PSA) levels

The total PSA levels were determined using the Prostate Specific Antigen (PSA) ELISA Kit and based on instructions of the manufacturer (Sigma-Aldrich, SA). The absorbance was read at 450 nm with a MTP reader (Offenburg, Germany).

Statistical analysis

Statistical analysis was performed using R statistical software with Agricolae package version 3.4.3 (R studio, Inc, Vienna, Austria). Normality of the data was assessed using the Shapiro Wilk test at a statistical significance level of ($P < 0.05$). Differences among group means were done using Tukey's test. Data were expressed as mean ± standard error (Bunn, 2008). Graphs were plotted using MedCalc for windows version 10 (MedCalc Software, Ostend, Belgium).

Results

Cell viability measured as mitochondrial dehydrogenase activity

Figures 1A, B and C show the effects of treating LNCaP and RWPE-1 cells with 125-50 000 µg/mL fermented rooibos for 72 hours.

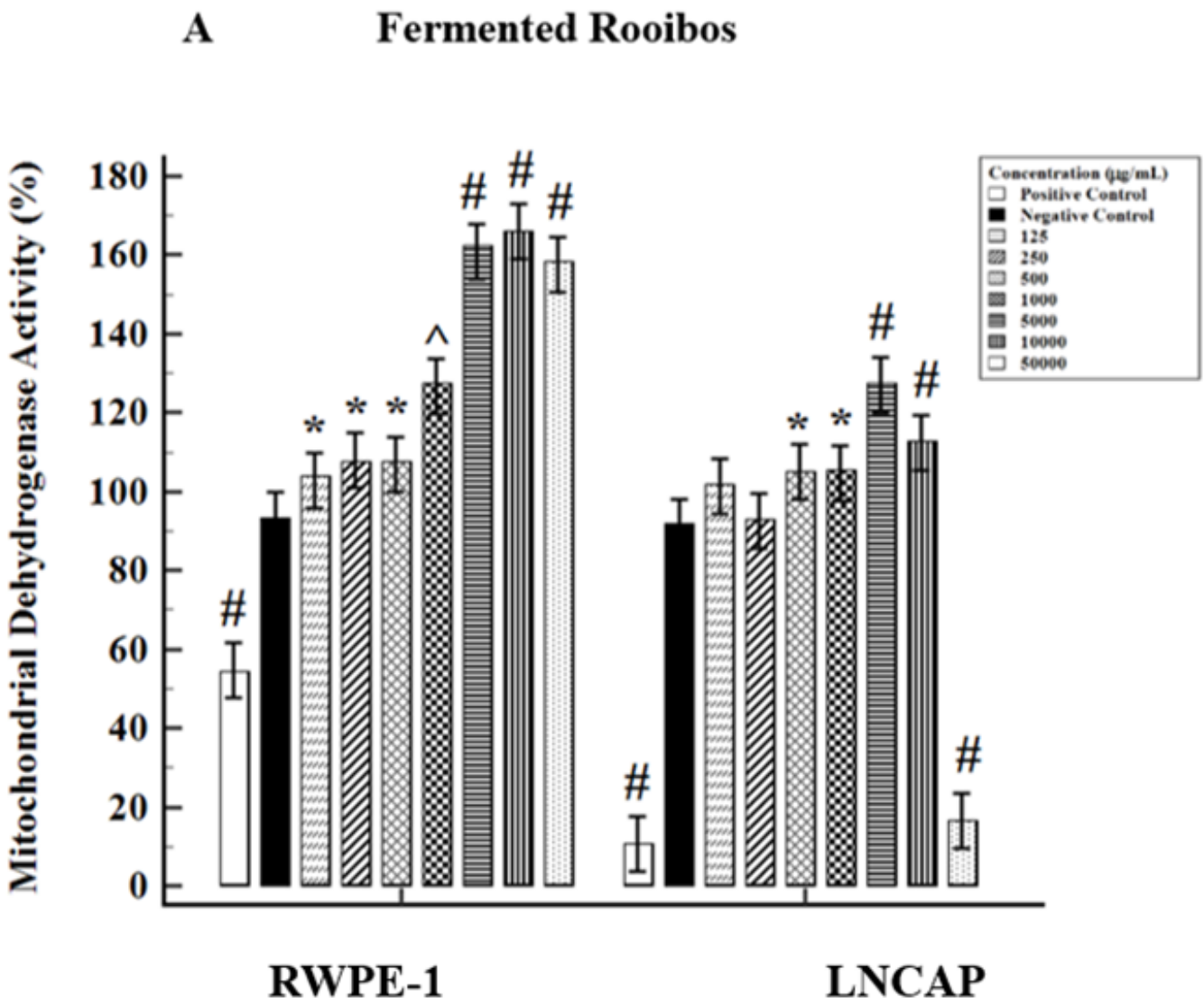
LNCaP cells treated with 125-250 µg/mL FR showed non-significant ($P > 0.05$) dose-dependent increase in mitochondrial dehydrogenase (mDHG) activity compared to its specific negative control. A further increase in the dosage of FR (500-1 000 µg/mL) promoted significant ($P < 0.05$) increase in mDHG activity which continued to significantly ($P < 0.001$) increase at 5 000 – 10 000 µg/mL FR and consequently promoted a significant ($P < 0.001$) drop in cell viability at 50 000 µg/mL FR compared to its specific negative control (Figure 1A).

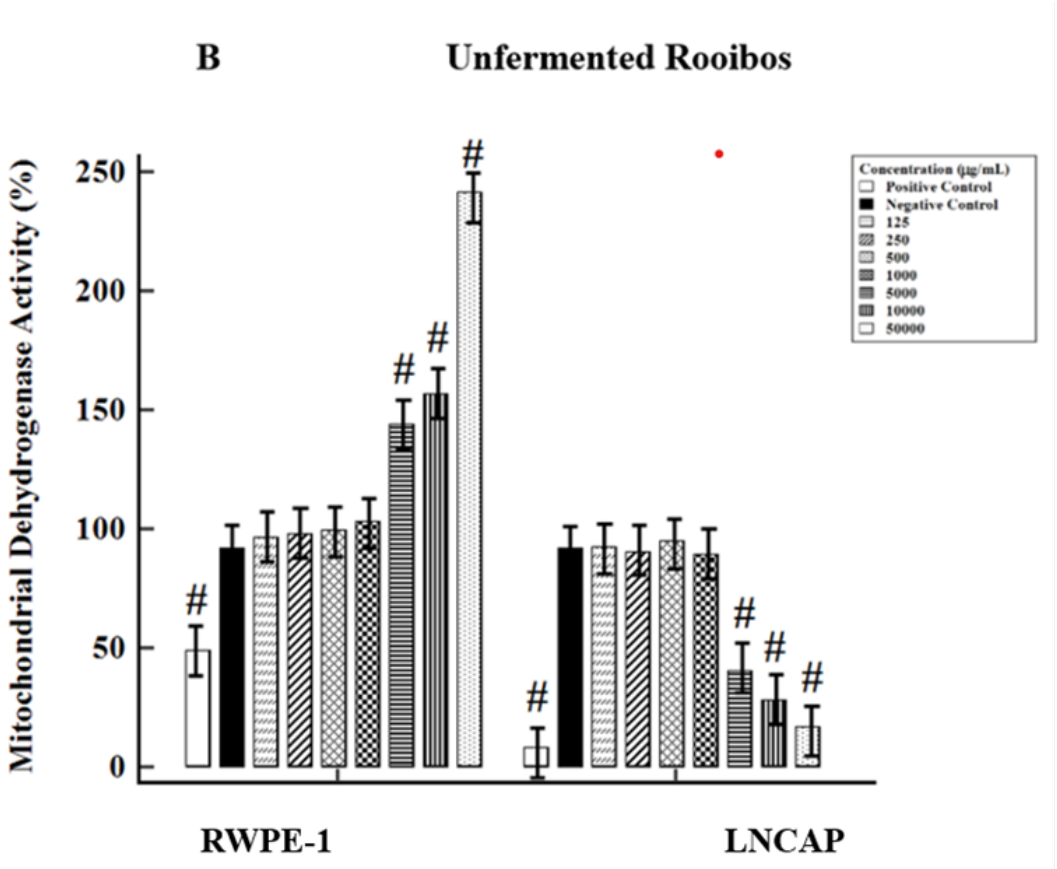
Meanwhile, RWPE-1 cells treated with 125 -500 µg/mL FR showed significant ($P < 0.05$) increase in mitochondrial dehydrogenase activity compared to its specific negative control. The mDHG activity continued to significantly ($P < 0.01$) increase at 1 000 µg/mL FR before inducing a further significant ($P < 0.001$) increase in mDHG activity at 5 000 – 50 000 µg/mL FR compared to its specific negative control. Interestingly, the mitochondrial dehydrogenase activity of RWPE-1 cells was significantly ($P < 0.05$) higher compared to LNCaP cells. Briefly, note that the cell death control (PC, 6% DMSO) caused a significant ($P < 0.001$) decrease in mDHG activity compared to the negative control (Figure 1A).

In reference to figure 1B, treatment of LNCaP cells with 125-1 000 µg/mL unfermented rooibos displayed a mitochondrial dehydrogenase activity that was not different from that of the negative control. However, at 5 000 -50 000 µg/mL UR showed significant ($P < 0.001$) concentration-dependent drop-in mitochondrial dehydrogenase activity compared to its specific negative control. Interestingly, RWPE-1 cells treated with 125 – 1 000 µg/mL UR showed mDHG activity that was comparable to the negative control; however, at 5 000 - 50 000 µg/mL UR, RWPE-1 cells showed significantly ($P < 0.001$) increased mitochondrial dehydrogenase activities thereby reflecting survival of the normal prostate cells (Figure 1B).

LNCaP cells treated with 125 µg/mL ASP (Figure 1C) showed mitochondrial dehydrogenase activity that was comparable to its specific negative control. However, treatment with 250 and 500 µg/mL ASP promoted significantly ($P < 0.01$) increased mitochondrial dehydrogenase activity, and this respiratory process continued to significantly ($P < 0.001$) increase when the cells were treated with 1 000 – 5 000 µg/mL ASP. Interestingly, the mitochondrial dehydrogenase activity significantly ($P < 0.05$) and ($P < 0.001$) dropped following treatment with 10 000 and 50 000 µg/mL ASP, respectively, when compared with its specific negative control.

Treatment of RWPE-1 cells with 125 µg/mL ASP promoted mitochondrial dehydrogenase activity that was comparable to its specific negative control. However, a significant ($P < 0.05$) increase in mDHG activity was observed at 250 µg/mL ASP. Consequently, the mDHG activity significantly ($P < 0.01$) increased at 500 - 1 000 µg/mL ASP compared to the negative control. This respiratory process eventually significantly ($P < 0.001$) increased following treatment with 5 000 and 10 000 µg/mL ASP before eventually dropping at 50 000 µg/mL ASP. Of special note, is the significant ($P < 0.001$) drop in mDHG activity of both LNCaP and RWPE-1 cell lines when treated with 50 000 µg/mL ASP when compared with their respective negative controls. Generally, the mDHG activities of both cell lines treated with 50 000 µg/mL ASP were comparable to those of their specific positive controls (6% DMSO) (Figure 1C).


A
Fermented Rooibos
Mitochondrial Dehydrogenase Activity (%)
180
160
140
120
100
80
60
40
20
0
Concentration (µg/mL)
Positive Control
Negative Control
125
250
500
1000
5000
10000
50000
RWPE-1
LNCAP



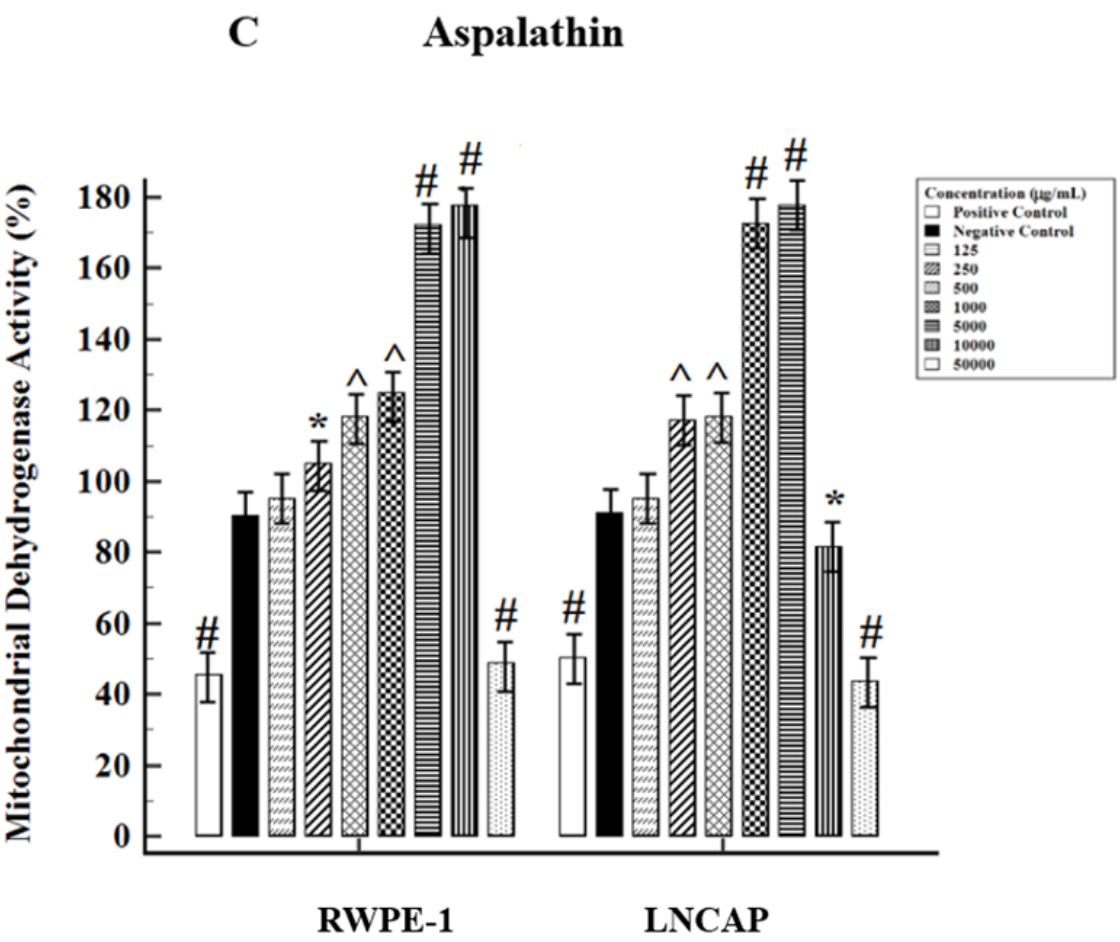


Figure 1: Cell viability expressed as mitochondrial dehydrogenase activity of RWPE-1 and LNCaP cells. (A) Fermented rooibos (B) Unfermented rooibos, (C) Aspalathin. Cells treated for 72-hours. Values represent mean ± SD n=9. Negative control = medium; Positive control = 6% DMSO. *P < 0.05; ^P < 0.01; #P < 0.001.

Morphometric studies

Figure 2 shows representative micrographs of RWPE-1 cells (A1 – 7) and LNCaP cells (B1-7) showing the morphology of RWPE-1 and LNCaP cells following treatment with *A . linearis* for 72 hours. Lower concentrations (125 -500 µg/mL) of fermented rooibos, show benign (RWPE-1) and malignant prostate cells that are normally dividing with cell numbers that are comparable to those of their respective negative control. However, at concentrations higher than 1 000 µg/mL, malignant (LNCaP) prostate cells (B5) appeared shrunk, irregular or round in shape and their cell numbers were reduced due to detachment and death whilst benign (RWPE-1) prostate cells did not show any change in morphology. NOTE: Aspalathin and fermented rooibos micrographs are not shown as they appeared similar to the unfermented rooibos.

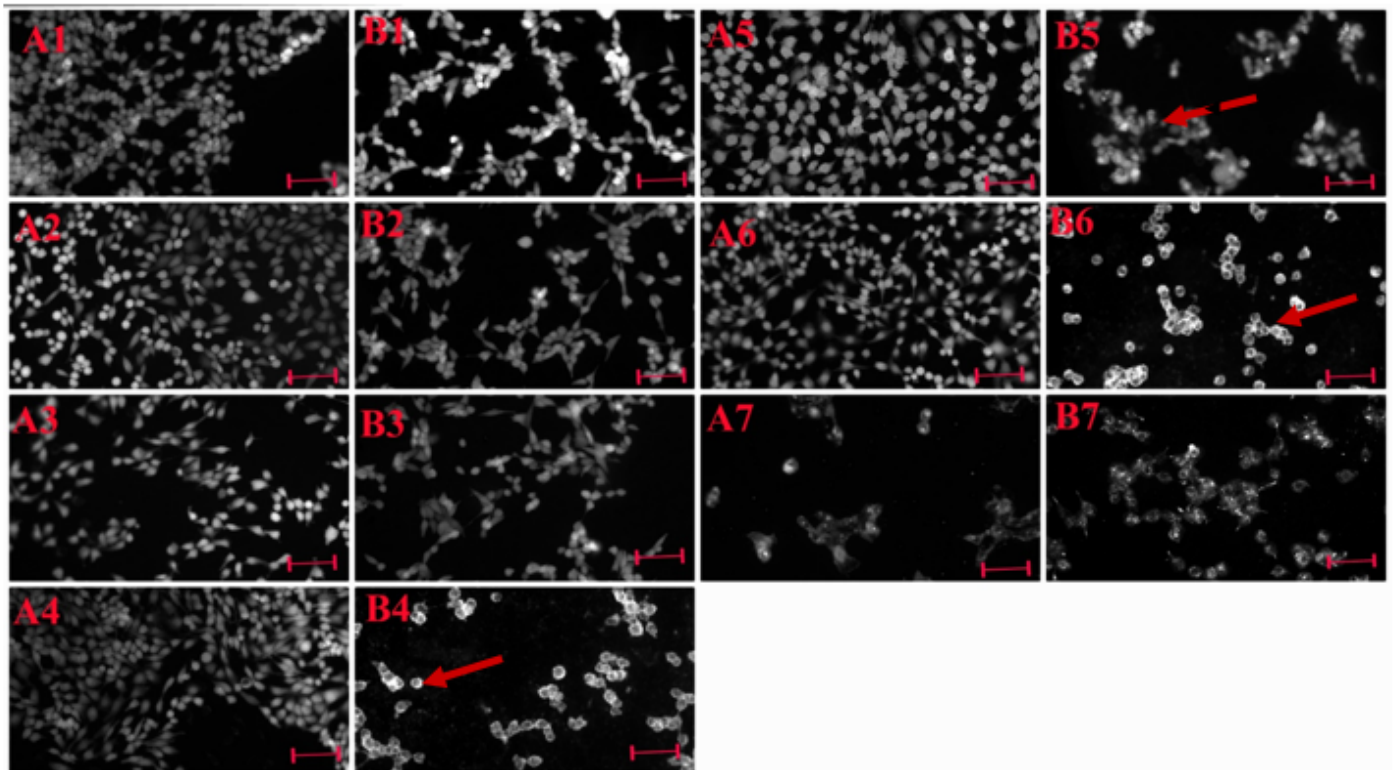


Figure 2: A comparison of ROS levels in RWPE-1 (A1-7) and LNCaP (B1-7) cells following exposure to rooibos for 72 hours.

(A1 & B1) Negative control (medium), (A2 & B2) 125 µg/mL, (A3 & B3) 250 µg/mL, (A4 & B4) 500 µg/mL, (A5 & B5) 1 000 µg/mL, (A6 & B6) 5 000 µg/mL, (A7 & B7) 10 000 µg/mL. Arrows illustrate cells that are swollen and have lost adhesion to other surrounding cells. Cells were stained with 7 µM CM 5, 6 CM H_2DCFDA. Magnification: 100x, Bar = 50 µm.

Reactive oxygen species (ROS)

With relation to the amount of ROS in benign RWPE-1 and malignant LNCaP prostate cells following incubation with FR, UR or aspalathin, all rooibos extracts stimulated higher ROS levels in LNCaP cells compared with the RWPE-1 cells (Figures 3A, B and C).

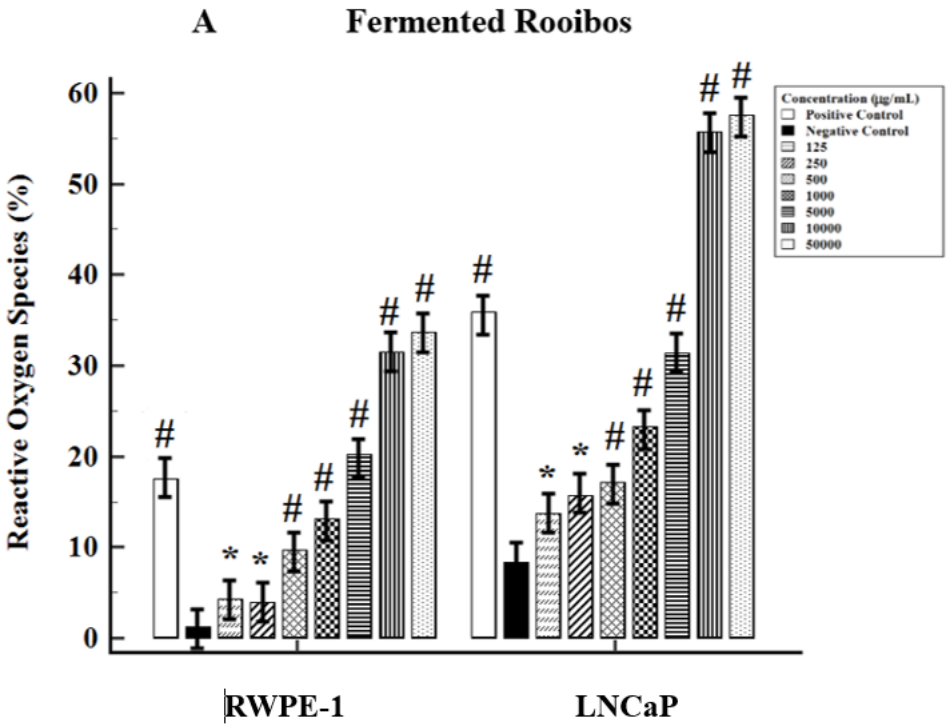
Exposure of LNCaP cells to 125 – 250 µg/mL FR led to a concentration-dependent significant ($P < 0.05$) increase in ROS levels compared to its specific negative control. However, a further increase in FR concentration promoted significantly ($P < 0.001$) increased ROS levels at all concentrations used. In the same assay, treatment of RWPE-1 cells with 125 – 250 µg/mL FR led to significantly ($P < 0.05$) increased mDHG activity, a biochemical process that continued to significantly ($P < 0.001$) increase at all subsequent concentrations used compared to its specific negative control (Figure 3A)

LNCaP cells treated with 125 - 250 µg/mL UR generated ROS levels that were comparable to those of its specific negative control. However, significantly ($P < 0.05$) increased ROS levels were generated in cells treated with 500 µg/mL UR. Of noteworthy, is the significantly ($P < 0.001$) increased ROS levels in LNCaP cells treated with 1 000 - 50 000 µg/mL UR (Figure 3B).

In the same assay, RWPE-1 cells treated with 125 µg/mL UR generated ROS levels that were comparable to those of its specific negative control. However, treatment with 250 – 500 µg/mL UR resulted in significantly ($P < 0.01$) increased ROS

levels that subsequently increased significantly ($P < 0.001$) upon treating RWPE-1 cells with 1 000 - 50 000 µg/mL UR compared to the negative control.

Exposure of LNCaP cells to 125 – 50 000 µg/mL ASP generated significantly ($P < 0.001$) increased levels of ROS compared with its specific negative control, however, no ROS levels were reported at 50 000 µg/mL ASP as all LNCaP cells were died. In the same assay, RWPE-1 cells, 125 µg/mL ASP produced non – significant ($P > 0.05$) increase in ROS levels compared with its specific negative control. However, 250 µg/mL ASP produced significantly ($P < 0.01$) increased levels of ROS which eventually significantly ($P < 0.001$) increased upon treatment with 500 – 10 000 µg/mL ASP compared with the negative control. Of noteworthy, RWPE-1 cells did not survive at 50 000 µg/mL ASP and no ROS levels were observed (Figure 3C).



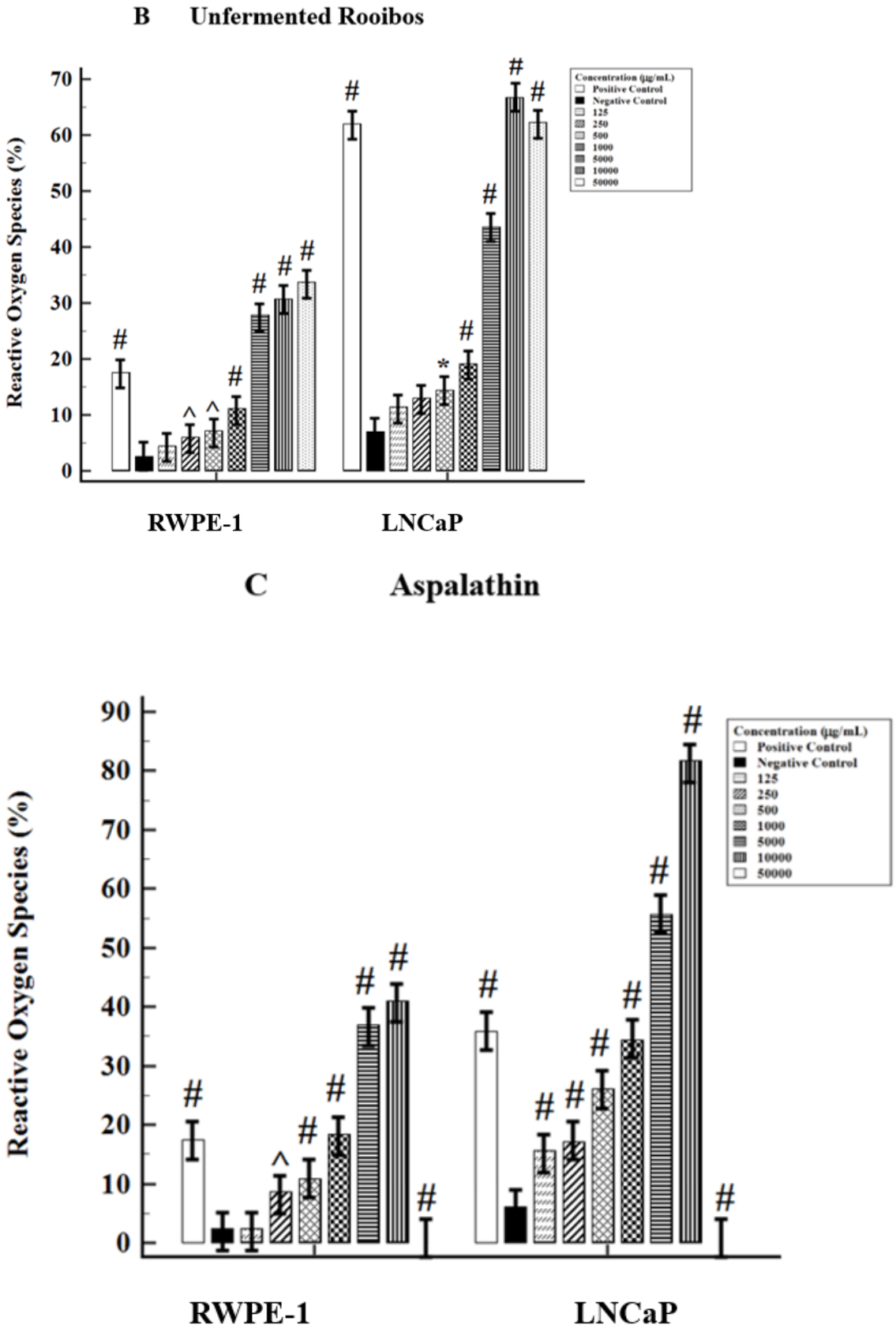


Figure 3: ROS production in benign (RWPE-1) and malignant (LNCaP) prostate cells following exposure to fermented rooibos (A), unfermented rooibos (B) or aspalathin (C) for 72 hours using flow cytometry. Values represent mean ± SD; n=9. PC, Positive control; 50 µM H2O2 for LNCaP and 100 µM H2O2 for RWPE-1. Negative control = medium; *$P < 0.05$; ^$P < 0.01$; #$P < 0.001$.

Apoptosis

A 72-hour incubation of malignant LNCaP with 1 000 µg/mL FR, UR or ASP, produced significant

($P < 0.001$) increases in the percentage of apoptotic malignant LNCaP cells compared to its specific negative control. Here attention should be drawn towards unfermented rooibos; a tisane that produced the highest percentage of apoptotic deaths compared to FR or ASP. On the contrary, treatment of RWPE-1 cells with 1 000 µg/mL FR, UR or ASP, produced significantly ($P < 0.001$) reduced percentage of apoptotic RWPE-1 cells compared to its specific negative control.

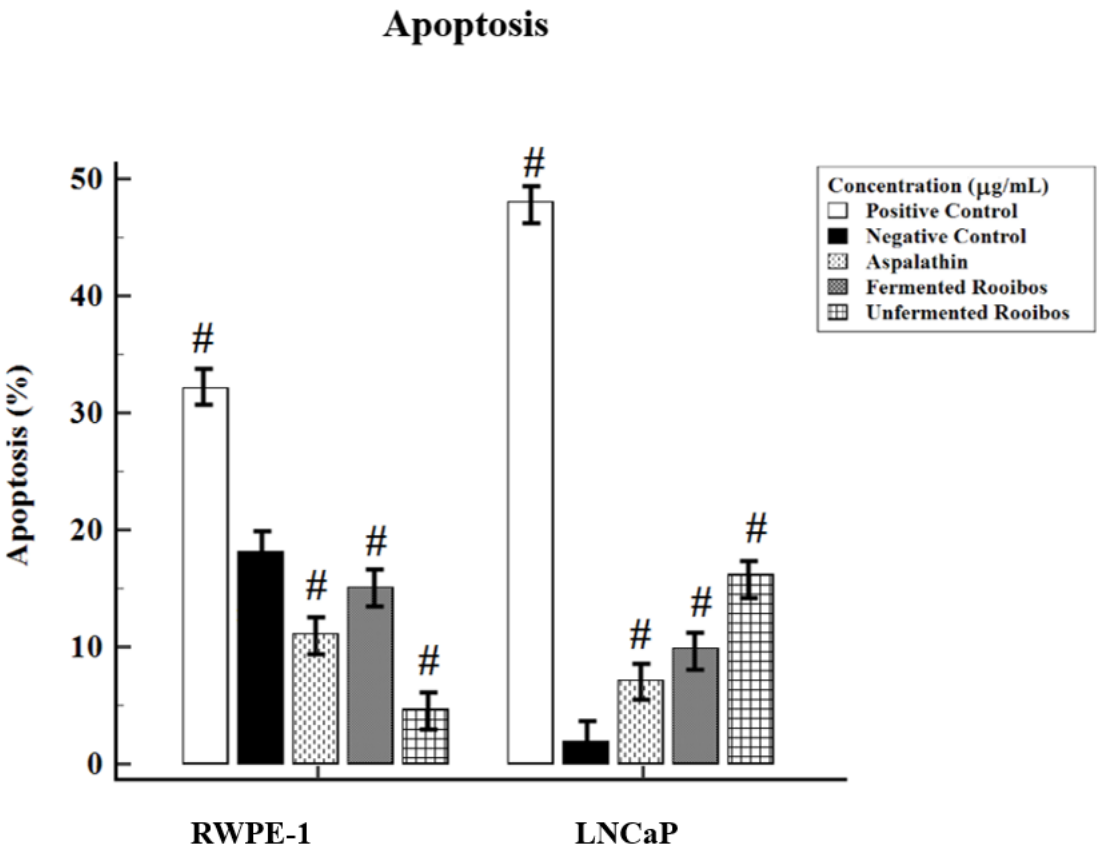


Figure 4: The apoptotic effect of 1000 µg/mL FR, UR and aspalathin on benign RWPE-1 and malignant (LNCaP) prostate cells following 72-hour incubation period, respectively, using flow cytometry. NC= negative control (medium); PC= positive control (6% DMSO); *$P < 0.05$; ^$P < 0.01$; #$P < 0.001$.

Necrosis.

A 72-hour incubation of LNCaP cells with 1 000 µg/mL FR, UR or ASP extracts resulted in an increased percentage of necrotic cells. In line with this, aspalathin and fermented rooibos produced non-significant ($P > 0.05$) increase in the percentage of necrotic cells compared with the negative control, however, unfermented rooibos produced significantly ($P < 0.001$) increased percentage of necrotic cells.

In the same assay, treatment of RWPE-1 cells with 1 000 µg/mL FR, UR or ASP extracts did not support necrosis. On the contrary, all the three rooibos tisanes produced significantly ($P < 0.001$) reduced percentage of necrotic cells compared with the negative control. Above all, the unfermented rooibos tisane appeared to give the greatest protection against necrosis to the benign RWPE-1 cells.

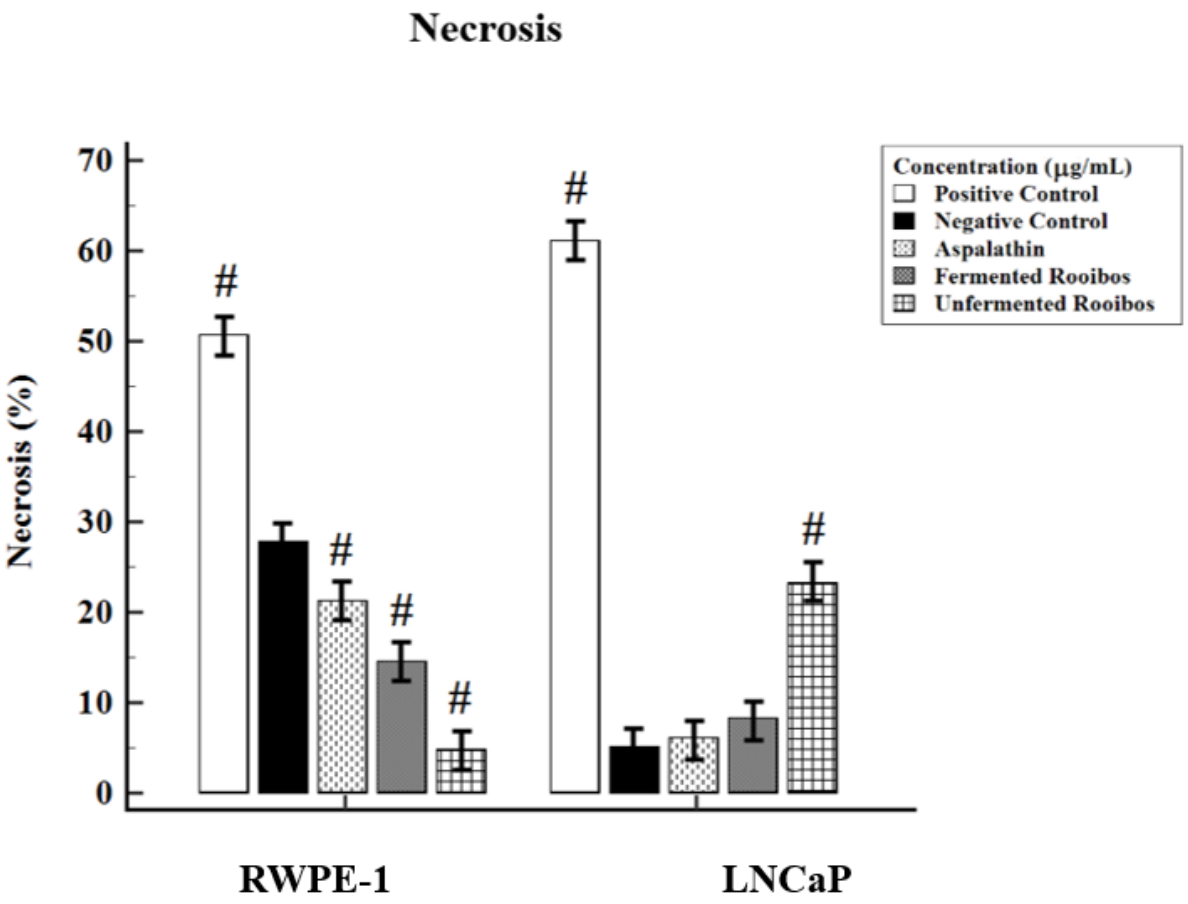


Figure 5: The necrotic effect of 1000 µg/mL FR, UR and aspalathin on benign RWPE-1 and malignant (LNCaP) prostate cancers following 72-hour incubation period, using flow cytometry. NC= negative control (medium); PC= positive control (6% DMSO). *$P < 0.05$; ^$P < 0.01$; #$P < 0.001$.

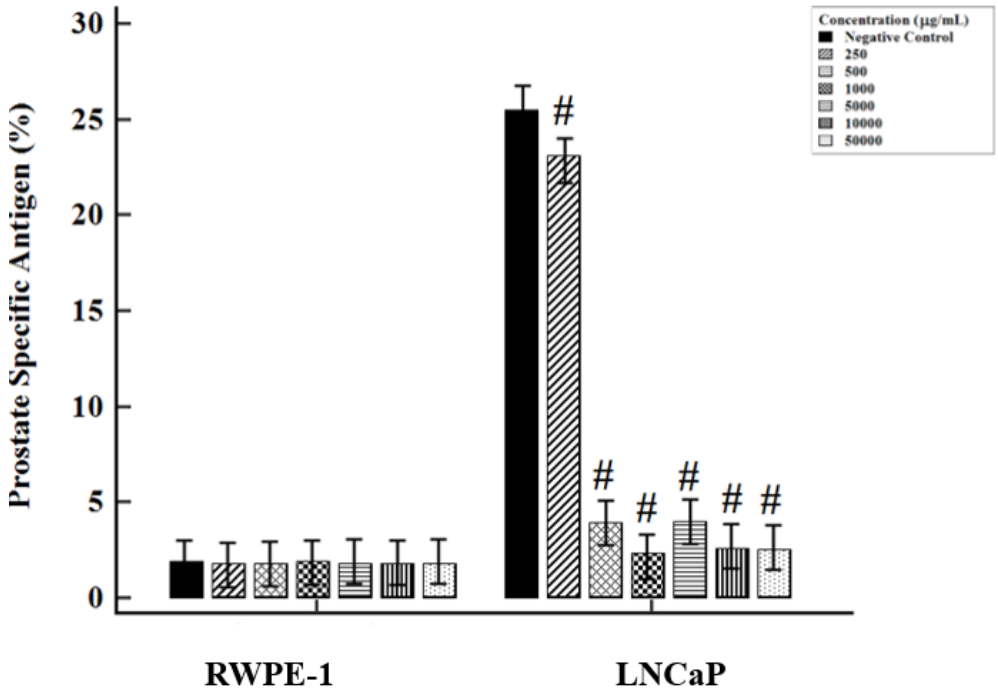
Prostate specific antigen (PSA)

Malignant LNCaP cells treated with 250 – 50 000 µg/mL FR displayed a significant ($P < 0.001$) decrease in the level of total PSA when compared to the negative control. In the same assay, RWPE-1 cells treated with 250 – 50 000 µg/mL FR, UR or ASP showed total PSA levels that were comparable to their specific negative control. Although the level of total serum PSA formed a plateau in benign RWPE-1, its concentration was much lower compared to the malignant LNCaP cells (Figures 6a).

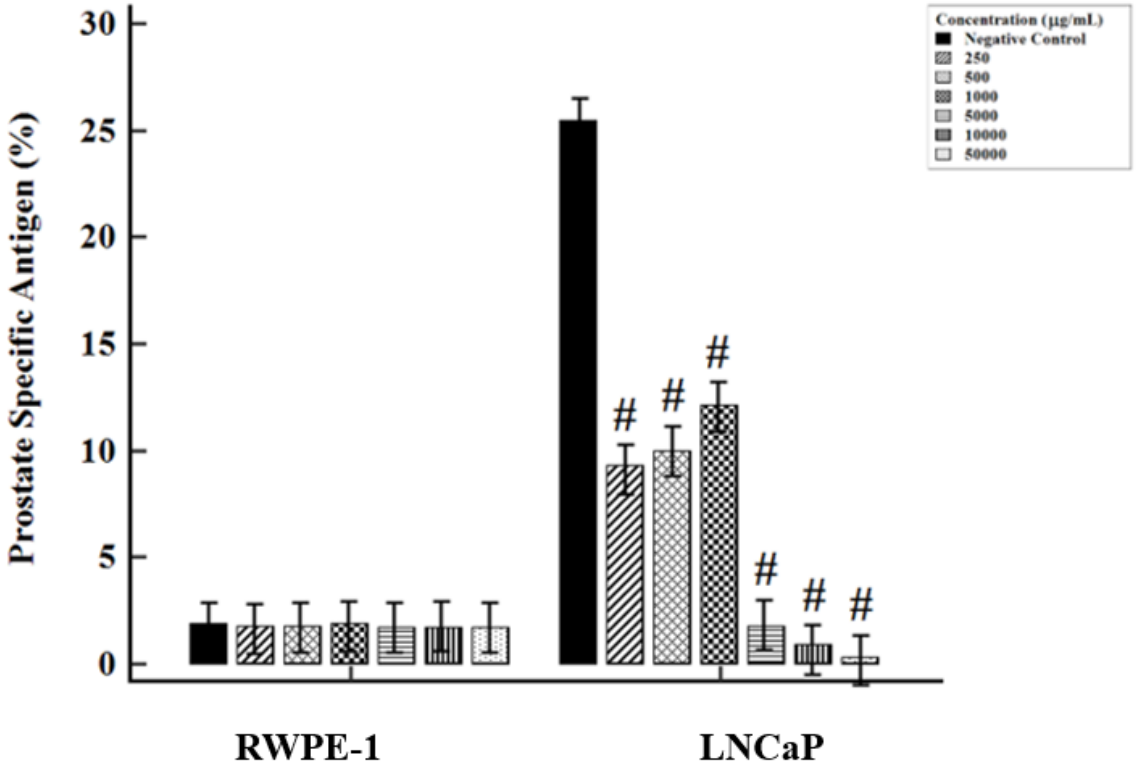
LNCaP cells treated with 250 -50 000 µg/mL UR showed significant ($P < 0.001$) drop in PSA levels compared with its specific negative control. Of noteworthy is the dose – dependent significant ($P < 0.001$) decrease in PSA levels from 5 000 µg/mL onwards. Meanwhile, there were no changes in the PSA levels for the treated RWPE-1 cells compared to their negative control (Figure 6b).

In figure 5c aspalathin treated LNCaP cells registered significantly ($P < 0.001$) decreased PSA levels compared with its specific negative control. Of noteworthy, is the absence of PSA levels in LNCaP cells treated with 50 000 µg/mL ASP. All LNCaP cells treated with this concentration died. Under the same conditions RWPE-1 showed PSA levels that were comparable to those of its specific negative control. Furthermore, all RWPE-1 cells treated with 50 000 µg/mL ASP were alive and produced total PSA levels that were comparable to the negative control.

A **Fermented Rooibos**



B **Unfermented Rooibos**



C Aspalathin

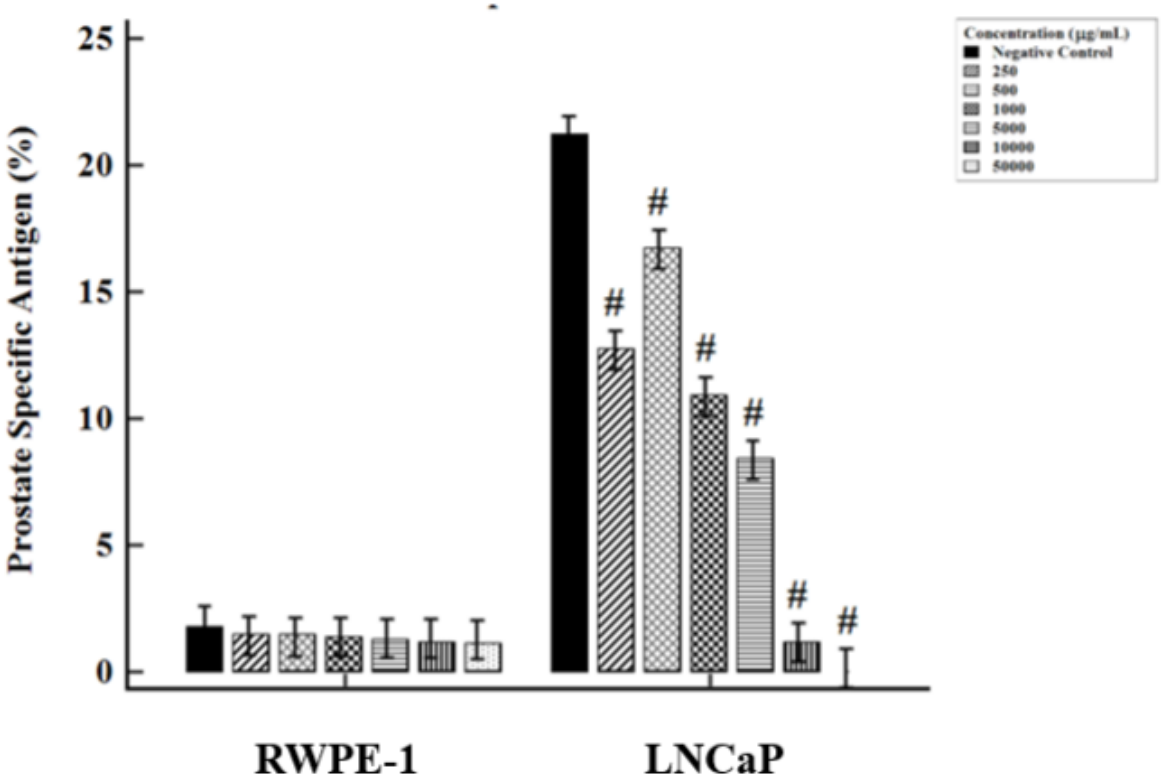


Figure 6: The effect of fermented, unfermented rooibos and aspalathin on total serum PSA in malignant (LNCaP) and benign (RWPE-1) prostate cells after 72-hour incubation period. (A) Fermented rooibos (B) Unfermented rooibos (C) Aspalathin. Values represented are the mean ± SD of 7 samples. FR; fermented rooibos. UR; Unfermented rooibos. ASP; Aspalathin. NC = Negative Control (Medium): *$P < 0.05$; ^$P < 0.01$; #$P < 0.001$.

Discussion

This study has shown that fermented (FR) and unfermented rooibos (UR) extracts or their active chemical compound, aspalathin, promote oxidative stress in LNCaP cell lines primary to the generation of high levels of reactive oxygen species (ROS) with the potential of inducing death in malignant prostate cells whilst protecting the normal prostate cells from ROS induced oxidative stress and death. The difference in extent of cell viability measured as mitochondrial dehydrogenase activity between the LNCaP and RWPE-1 cells observed herein point at a possible pro-oxidant effect of rooibos on malignant prostate cells and antioxidant effect in normal prostate cells. In line with this, ROS generated in LNCaP cells produced significantly increased levels of apoptotic and necrotic cells compared to RWPE-1 cells. In this regard, LNCaP micrographs showed malignant prostate cells that are shrunk, irregular or round in shape with reduced cell numbers compared to their specific negative control meaning that unfermented rooibos extracts were cytotoxic to the malignant (LNCaP) prostate cells (Figure 2; B1-B7).

Interestingly, in RWPE-1 cells, UR induced significantly reduced apoptotic or necrotic deaths followed by FR and ASP. All RWPE-1 micrographs show normal adherent and dividing cells with flat polygonal shapes with cell numbers that are comparable to those of their specific negative control, meaning that rooibos did not affect the viability of normal (RWPE-1) prostate cells (Figure 2; A1-A6). These results are suggesting potential therapeutic effect of rooibos on malignant prostate cells and protective effects on normal prostate cells.

Reports indicate lower total polyphenol levels in FR compared to UR primarily due to the oxidation process that is involved with fermentation which promotes structural and enzymatic changes which may reduce the original aspalathin content to 7% (Canda, Oguntibeju and Marnewick, 2014). Probably, this is the more reason that unfermented rooibos exerted significantly increased cytotoxic effects on LNCaP cells compared with fermented rooibos or aspalathin.

The most common method by which ROS kill cancerous cells is by activation of programmed cell death which can be initiated by mitochondrial (intrinsic), or death receptor (extrinsic) pathways which result in the formation of apoptotic bodies that eventually get eliminated by phagocytes. Though speculative, the ROS levels generated by rooibos herein; may have activated the extrinsic apoptotic pathway leading to apoptotic and necrotic deaths of malignant LNCaP cells (Villalpando-Rodriguez and Gibson, 2021). In this regard, rooibos may have initiated the activation of death receptors belonging to the tumor necrosis factor (TNF) family: TNFRSF1A, FAS (CD95, or APO-1), TNFSF10/TRAIL receptors TNFRSF10A/DR4, TNFRSF10B/DR5, TNFRSF25/DR3 and TNFRSF21/DR6 (Verbrugge, Johnstone and Smyth, 2010). And signaling through CD95 and TRAIL-R1/-R2 ligands led to the formation of a death-inducing signaling complex (DISC) that activated the initiator caspases 8 and 10. Activated caspases 8 and 10 may have activated the effector caspases 3, 6 and 7 and consequently leading to apoptotic

deaths observed herein (Ali and Kulik, 2021).

In the intrinsic pathway, intracellular ROS generated in LNCaP cells may have activated proapoptotic proteins BAK and BAX of the BCL-2 family of proteins which may have promoted mitochondrial outer membrane permeabilization (Villalpando-Rodriguez and Gibson, 2021). This permeabilization may have led to the release of apoptotic molecules like apoptosis inducing factor (AIF), SMAC/DIABLO, or cytochrome c. Furthermore, cytosolic cytochrome c upon binding to apoptotic protease-activating factor-1 (APAF-1) may have led to its oligomerization and its recruitment of procaspase 9 thereby forming the apoptosome complex that mediates the activation of an initiator caspase. In this regard, procaspase 9 within the apoptosome may have been activated and liberated from the complex leading to the activation of executioner caspases 3 and or caspase 7 (Redza-Dutordoir and Averill-Bates, 2016).

Elsewhere, UR containing 12.78% aspalathin suppressed the expression of proteins involved in phosphoinositide 3-kinase (PI3K)-Akt signalling, hence preventing the survival of prostate cancer cells (Wang et al., 2022). Additionally, UR suppressed the androgen receptor (AR), phospho-AR (Ser81), cyclin-dependent kinase 1 (Cdk1), cMyc and Bcl-2 thereby dysregulating the cell cycle and increasing the expression of apoptotic proteins (Wang et al., 2022).

Besides the aforesaid apoptotic mechanisms, UR extracts showed a decline in PCa cell population in the S phase but increased the same in the G_2/M phase. In this regard, UR showed the potential of disrupting DNA synthesis and prematurely forcing prostate cancer cells into mitosis and death. Additionally, UR extracts suppressed Akt1, phospho-Akt Ser473, Cdc2, Bcl-2, TRAF4 and Aven but increased activated Caspase 3, cytochrome c, and p27Kip1 proteins (Shih Han Huang et al., 2019).

A phytochemical analysis of rooibos has shown the presence of polyphenols such as flavanols, flavones, flavanones, dihydrochalcones, aspalathin and nothofagin, gallic acid (Coetzee et al., 2014). Elsewhere, rooibos through polyphenol gallic acid; promoted ROS-dependent apoptosis in LNCaP cells. To this effect, autoxidation of gallic acid generated significant levels of hydrogen peroxide (H_2O_2) and superoxide radicals (O_2-) which were implicated for the ROS induced death of the malignant prostate cells (Russell et al., 2012). Additionally, rooibos through flavone acacetin, inhibited cell growth and induced apoptosis in human prostate cells (DU145) by binding directly to signal transducer and activator of transcription 3 (STAT3) and this prevented both its phosphorylation at tyrosine 705 residue and translocation to the nucleus hence inducing apoptosis (Yun et al., 2021).

The results on ROS, apoptosis and necrosis assay observed in this study, suggest the interpretation that FR, UR and ASP, created differential oxidative environments between malignant (LNCaP) prostate cells and benign (RWPE-1) cells through exploitation of a compromised redox homeostasis in the tumor cells. Reports have been made to the effect that prostate cancer cells generate more ROS and NADPH oxidase (Nox) enzymes compared to the normal prostate tissue due to the presence of ectopic sources of ROS in prostate cancer cells. The sources include various forms of Nox (Nox1, Nox2, Nox3, Nox4 and Nox5) which are not present in normal prostate cells (Miyata et al., 2017). Further to this, in tumor cells O_2^- is mainly generated by mitochondrial respiratory chain (by complex I and III). On the contrary, in normal cells there is low production of O_2^- and the cytosolic NADPH oxidase is responsible for the generation of ROS (Laurent et al., 2005).

In a previous study, plant polyphenols have been reported to possess both antioxidant as well as pro-oxidant properties (Azmi et al., 2006). Further to this, the pro-oxidant action of these polyphenols is responsible for the mobilization of endogenous copper ions and the consequent pro-oxidant action leads to the generation of super oxide anion and hydrogen peroxide which are further converted into hydroxyl radicals (Azmi et al., 2006). Compromised superoxide dismutase and catalase activity in malignant cells results in diminished conversion of superoxide anion and H_2O_2 to H_2O and O_2 and hence the increased levels of ROS in cancer cells. These high ROS levels are responsible for the cytotoxic actions of the polyphenols on cancer cells (Hadi, 2000).

Besides inducing apoptosis and necrosis in malignant LNCaP cells FR, UR and aspalathin produced a concentration-dependent decrease in total serum PSA concentration whereas in RWPE-1 cells, the treated samples produced no observable change in total serum PSA levels (figure 5a, b and c). And the concentration-dependent decrease in PSA levels observed in this study; represents a direct relationship with shrinkage of prostate tumor (Daisuke Obinata et al., 2020) and death of the cancerous cells. Interestingly, rooibos did not did not kill the benign prostate cells as reflected by the absence of an observable change in PSA levels, normal shape of cells and cell numbers on the micrographs which suggests the interpretation of rooibos' lack of cytotoxicity against normal prostate cells. Similarly, a reduction in serum levels of PSA, HGF, IGF-1, IGFBP-3 and VEGF have been reported in men with prostate cancer after a brief treatment with EGCG (Polyphenon E) with no elevation of liver enzymes (McLarty et al., 2009).

The secretion of Prostate-Specific Antigen (PSA) is regulated by the activation of the Androgen Receptor (AR). Elsewhere, reduced androgen receptor-activated PSA levels have been reported following LNCaP exposure to rooibos in the presence or absence of dihydrotestosterone (DHT). In line with this, suggestions have been made to the effect that rooibos binds to androgen receptor (AR) or oestrogen receptor-β (ERβ) expressed in LNCaP cells thereby inhibiting its synthesis. This rooibos inhibitory effect, enhances less activation of the AR by DHT and subsequently less AR-activated PSA secretion (Jia et al., 2003).

The results presented herein are consistent with the cell specific effects of rooibos or aspalathin to the extent that it may induce cell death of malignant prostate cells through increased reactive oxygen species (ROS) production and may protect benign prostate cells from ROS induced stress.

This study suggests cytotoxic effects of unfermented rooibos on malignant LNCaP cells and protective effects on benign RWPE-1 cells against ROS induced stress. The decrease in total serum PSA seems to demonstrate possible therapeutic effects of *A. linearis* on malignant LNCaP prostate cells and possible maximum health benefits from total polyphenol content.

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